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Salmonella enterica Serovar Typhimurium Binds to HeLa Cells via Fim-Mediated Reversible Adhesion and Irreversible Type Three Secretion System 1-Mediated Docking

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Salmonella enterica Serovar Typhimurium Binds to HeLa Cells via Fim-Mediated Reversible Adhesion and Irreversible Type Three Secretion System 1-Mediated Docking[▽]

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The food-borne pathogen *Salmonella enterica* serovar Typhimurium invades mammalian epithelial cells. This multistep process comprises bacterial binding to the host cell, activation of the *Salmonella* type three secretion system 1 (T1), injection of effector proteins, triggering of host cell actin rearrangements, and *S. Typhimurium* entry. While the latter steps are well understood, much less is known about the initial binding step. Earlier work had implicated adhesins (but not T1) or T1 (but not other adhesins). We have studied here the *Salmonella* virulence factors mediating *S. Typhimurium* binding to HeLa cells. Using an automated microscopy assay and isogenic *S. Typhimurium* mutants, we analyzed the role of T1 and of several known adhesins (Fim, Pef, Lpf, Agf, and Shd) in host cell binding. In wild-type *S. Typhimurium*, host cell binding was mostly attributable to T1. However, in the absence of T1, Fim (but not Pef, Lpf, Agf, and Shd) also mediated HeLa cell binding. Furthermore, in the absence of T1 and type I fimbriae (Fim), we still observed residual binding, pointing toward at least one additional, unidentified binding mechanism. Dissociation experiments established that T1-mediated binding was irreversible (“docking”), while Fim-mediated binding was reversible (“reversible adhesion”). Finally, we show that noninvasive bacteria docking via T1 or adhering via Fim can efficiently invade HeLa cells, if actin rearrangements are triggered *in trans* by a wild-type *S. Typhimurium* helper strain. Our data show that binding to HeLa cells is mediated by at least two different mechanisms and that both can lead to invasion if actin rearrangements are triggered.

Salmonella enterica subspecies 1 serovar Typhimurium is a common cause of food-borne disease. Central to the pathogenesis of *S. Typhimurium* is the ability to invade epithelial cells (45, 53, 58). Host cell invasion is a multistep process that is initiated by pathogen binding to the host cell, activation of the type three secretion system 1 (T1), and insertion of a T1 conduit (“translocon”) into the host cell membrane. After this, T1 effector proteins are translocated into the host cell, which in turn trigger actin rearrangements and internalization (invasion) of the pathogen into the host cell (see Fig. 1A). The latter steps have received much attention. In contrast, the binding step preceding invasion is not completely understood and may involve reversible and irreversible mechanisms, termed “reversible adhesion” and “docking” in the present study.

Several *Salmonella* virulence factors have been implicated in binding to host surfaces. Fimbrial adhesins are well known mediators of host cell binding and obvious candidates for contributing to the initial binding step leading to host cell invasion. The *S. Typhimurium* LT2 genome encodes 13 adhesin gene clusters (14, 54), several of which have been analyzed functionally in the past. These include type I fimbriae (Fim) (8, 12, 44), plasmid-encoded fimbriae (Pef) (2, 19, 50), long polar fimbriae

(Lpf) (1), thin aggregative fimbriae (Agf) (23, 55, 67), the adhesin ShdA (39), and also *Salmonella* pathogenicity island 4 (SPI-4), encoding the YadA-like adhesin SiiE (21). SiiE enhances T1-mediated invasion into polarized epithelial cells but not into fibroblasts and other nonpolarized cells (20). Thus, particular adhesins may contribute to invasion of particular types of host cells and therefore, the required adhesins need to be established for each cell type of interest.

Recently, the T1 apparatus itself was found to mediate host cell binding (42). This binding phenotype of T1 had escaped attention before, since some studies used culture conditions unfavorable for T1 expression. In other experiments, the massive T1-mediated actin rearrangements accompanying membrane ruffling and host cell invasion, which occur within minutes after the initial binding, may have precluded detailed analysis of the binding step (17, 18, 22; reviewed in references 45, 53, and 58). These latter phenotypes are triggered by translocated T1 effector proteins, which stimulate actin polymerization either directly or indirectly. SipA can directly engage and polymerize actin (43, 46, 71), whereas SopE, SopE2, and SopB lead to the activation of Rho-GTPases (28, 51, 52, 66), which in turn signal to a powerful actin nucleator, the Arp2/3 complex (10). This elicits the typical “membrane ruffles” on the surface of the host cell (see Fig. 1B), which are also the sites of *S. Typhimurium* invasion. *S. Typhimurium* mutants with an intact T1 apparatus, but lacking SopE, SopE2, SipA, and SopB, do not trigger actin rearrangements or membrane ruffling and are impaired in cellular invasion (48, 59, 70). These types of *S. Typhimurium* mutants facilitated the discovery of the binding phenotype of T1 (42). However, it had remained unclear

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TABLE 1. Strains and plasmids used

Original strain or plasmid name	Strain name used in the present study	Genotype	Source or reference
Strains (SL1344 background)			
SL1344, SB300	wt	Wild-type <i>S. Typhimurium</i>	34
M566	$\Delta 4$	$\Delta sipA \Delta sopBEE2$	15
SB161	$T1^-$	$\Delta invG$	37
M1915	$T1^- Fi^-$	$\Delta invG fimD::pGP704$	This study
M1913	$SipBCD^-$	$\Delta sopBEE2 sipA-sptP::aphT$	This study
M1914	$T1^- SipBCD^-$	$\Delta sopBEE2 \Delta invG sipA-sptP::aphT$	This study
M1910	$SipB^-$	$\Delta sopBEE2 sipB::aphT$	This study
M1911	$SipC^-$	$\Delta sopBEE2 sipC::aphT$	This study
M1912	$SipD^-$	$\Delta sopBEE2 sipD::aphT$	This study
M1304	$\Delta 4 SipA$	$\Delta sipA \Delta sopABEE2 sipA_{M45}$	60
M1930	$T1^- PefC^-$	$\Delta invG pefC::pSB377$	This study
M1929	$T1^- LpfC^-$	$\Delta invG lpfC::pSB377$	This study
M1931	$T1^- AgfB^-$	$\Delta invG agfB::pSB377$	This study
M1928	$T1^- ShdA^-$	$\Delta invG shdA::pGP704$	This study
M2955	$T1^- FimA^-$	$\Delta invG fimA::Km^r$	This study
M2957	$T1^- FimH^-$	$\Delta invG fimH::Km^r$	This study
M2953	$\Delta 4 FimD^-$	$\Delta sipA \Delta sopABEE2 fimD::pGP704$	This study
M2954	$\Delta 4 FimA^-$	$\Delta sipA \Delta sopABEE2 fimA::Km^r$	This study
M2956	$\Delta 4 FimH^-$	$\Delta sipA \Delta sopABEE2 fimH::Km^r$	This study
M915	$FimD^-$	$fimD::pGP704$	This study
Plasmids			
pM975		Intracellular GFP expression	27
pM965		Constitutive GFP expression	64

whether the $T1$ -mediated cell binding represented a true intermediate of the invasion process, whether binding was reversible or irreversible, and whether other adhesins might also contribute.

In the present study, we analyzed the binding of *S. Typhimurium* to HeLa cells, a commonly used cell line for studying the mechanism of *Salmonella*-host cell invasion. We have devised an automated microscopy assay for *S. Typhimurium* binding, confirmed a $T1$ -dependent binding, and demonstrated a role of the type I fimbriae. The binding mediated by the former is irreversible, whereas the latter is reversible. Nevertheless, both modes of binding could lead to host cell entry if actin rearrangements were induced. Thus, different modes of binding can initiate HeLa cell invasion by *S. Typhimurium*.

MATERIALS AND METHODS

Automated microscopy assay for *S. Typhimurium* binding. HeLa cells were seeded 24 h prior to infection in 96-well μ -Clear plates (half-size; Greiner) at 6,000 cells per well in Dulbecco modified Eagle medium (DMEM), 10% fetal calf serum (FCS). *S. Typhimurium* was diluted in 2-fold series in ice-cold DMEM; subsequently, 12 μ l of DMEM harboring sufficient bacteria to yield the indicated final multiplicity of infection (MOI) in a total volume of 72 μ l was used. After the indicated time of infection, the plates were emptied and washed three times with 50 μ l of DMEM, 10% FCS using a Wellmate (Matrix). Fixation was performed with 4% paraformaldehyde, 4% sucrose in phosphate buffer saline (PBS), followed by incubation in 20% sucrose in PBS overnight. *S. Typhimurium* was visualized by indirect immunofluorescence using a rabbit anti-*S. Typhimurium* antibody (Difco) and a fluorescein-5-isothiocyanate (FITC)-labeled goat anti-rabbit secondary antibody (Jackson). Nuclei were stained with DAPI (4',6'-diamidino-2-phenylindole) after a 5-min permeabilization step using 0.1% Triton X-100. See Table 1 for a complete list of the *S. Typhimurium* strains and plasmids used.

For inhibitor experiments, α -methyl-mannose was used (Sigma). Cells were preincubated with the inhibitor at the indicated concentration or the same concentration of glucose at 37°C for 10 min. Bacteria were preincubated with the same concentration of the inhibitor on ice. Afterwards, the cells were infected, and docking was analyzed as described above.

For the helper assays, strain M566, a $\Delta sipA \Delta sopBEE2$ mutant (referred to here as the $\Delta 4$ strain), and strain SB161, a $\Delta invG$ mutant (referred to here as the $T1^-$ strain), carrying plasmid pM975 for intracellular *gfp* expression, were used (59). *S. Typhimurium* was first allowed to associate with cells as described above, followed by three washes with DMEM, 10% FCS (see above), and either fixed and stained for determination of bound *S. Typhimurium* or incubated with 60 μ l of DMEM, 10% FCS and the indicated MOIs of the wild-type strain SL1344 (referred to here as the wt strain), $\Delta 4$, or $T1^-$ strains as indicated for 42 min at 37°C in 5% CO_2 . The wells were subsequently emptied, followed by 4 h of incubation in 50 μ l of DMEM, 10% FCS and gentamicin (400 μ g/ml), fixation, and incubation in 20% sucrose (see above). Nuclei were stained by using DAPI at 10 μ g/ml and 0.1% Triton X-100 for 7 min. Quantification of invasion was done as described for docking, except that the green fluorescent protein (GFP) signal produced by intracellular bacteria was used instead of indirect immunofluorescence (59).

For dissociation experiments, cells were preincubated with *S. Typhimurium* at the indicated MOIs for 12 or 60 min, followed by three washes as described above, and the following sequential incubation steps at 37°C in 5% CO_2 . After 10 min, the wells were emptied and refilled with either DMEM, 10% FCS and 100 μ M α -methyl-mannose or DMEM, 10% FCS and 20% sucrose to achieve the appropriate numbers of incubation steps and with 4% paraformaldehyde to terminate the assay. For the estimation of dissociation kinetics, trend lines were fitted using the power (exponentiation) option of the trend line function of Microsoft Excel and by fitting the parameters *a* and *b* to the curve $y = a \cdot x^b$. In this way, 27 original curves were fitted, and the decay parameter *b* was used to calculate the half-life in minutes. A statistical comparison of the 27 calculated half-lives was performed using the Mann-Whitney U test. For experiments evaluating the effect of the HeLa cell density on bacterial binding, the cells were seeded into 96-well plates and grown to obtain approximately 100, 250, 500, 750, 1,000, 2,000, 3,000, 5,000, 7,500, 10,000, or 15,000 cells per well, as described above. Cells were infected with the $\Delta 4$ strain, the $T1^-$ strain, and strain M1915, a $\Delta invG fimD::pGP704$ mutant (referred to here as the $T1^- Fi^-$ strain), respectively, at the indicated MOIs. Docking was measured as described above. The percentage of cells with associated *S. Typhimurium* is shown as a function of the number of nuclei detected within the four acquired microscopy images per well.

If not indicated otherwise, experiments have been performed in three independent experiments, and we report the medians and the standard deviations.

Automated image analysis. The percentage of cells with bound or internalized *S. Typhimurium* was determined by using automated microscopy and image analysis (59). Four images per well were acquired in the DAPI and FITC

channels using an Image Express microscope (Molecular Devices). Images were analyzed by using the open-source program CellProfiler (6). Nuclei were detected in the DAPI channel; spots were detected in the GFP channel using the IdentifyPrimAutomatic module. Nuclei were expanded to estimate the dimensions of the cells using the distance option of the IdentifySecondary module. This algorithm might in some cases not accurately determine cell borders and include some adjacent "empty" area. However, since binding to "empty" areas of a well was far less efficient than binding to cells, our results should not be significantly affected by this. Spots were also identified with the IdentifyPrimAutomatic module. Here, an optimal threshold was calculated by comparing images from non-infected and infected wells using a customized algorithm. This threshold was held constant throughout the experiment. Finally, spots and cells were superimposed using the Relate module. The largest area of overlap determined the allocation to a particular cell. In a postprocessing step after image analysis, cells carrying no spots were labeled as uninfected, cells carrying one or more spots were labeled as "infected" or "with bound bacteria." Details of the CellProfiler "pipeline" and our customized algorithms are available upon request. Overall, 5,000 to 14,000 cells were evaluated per well, allowing for a high precision of measurement. For replicate samples, a precision of >95% was consistently achieved; error bars in our data (see, for instance, Fig. 2B) are due to biological variability of bacteria and host cells. In all experiments performed, the fraction of "positive" cells (i.e., cells harboring associated or internalized bacteria) in noninfected control wells was negligible (<1%), confirming the high specificity of our assay. As a quality control, the number of nuclei, as well as many parameters of the image analysis, was monitored for each well. The cell number was found to be very stable in docking experiments: Under the conditions described in a typical experiment a median of 9,500 cells per well (standard deviation = 1,980) were detected. Within one experiment per 96-well plate, the standard deviation was even lower (8.1%).

To determine the relative affinities of *S. Typhimurium* for cells and for empty areas of the well, the area covered by cells was determined for each well using the cellular outlines, as calculated by the CellProfiler modules. Subsequently, the cell-free area of each well was calculated. In a conservative approach, the cellular outlines were expanded, and the area outside of these expanded cells, excluding the rim area of each image, was considered as cell-free. Finally, for each well the area calculated was divided by the number of spots located within the respective areas. Details of these algorithms are available upon request.

Statistical analysis. Nonparametric tests were used for all analyses. If sufficient biological replicates were available, a Mann-Whitney U test was performed, comparing individual data points for experimental samples and control samples (see, for example, Fig. 2B and 6). For the experimental results shown in Fig. 2C and 4, only three biological replicates were available. In this situation, a paired analysis (Wilcoxon signed-rank test) was performed comparing the experimental curve consisting of the median of the three biological replicates with a control curve. For the results shown in Fig. 8A, paired analysis of all biological replicates was performed (Wilcoxon signed-rank test). The programs Prism, Matlab, and SPSS were used for the statistical calculations. A *P* value of <0.05 was considered statistically significant.

High-resolution images. For high-resolution images, HeLa cells were seeded on glass coverslips for 24 h and infected with *S. Typhimurium* carrying plasmid pM965 for constitutive *gfp* expression at an MOI of 128 for 12 min. After fixation, *S. Typhimurium* samples were stained by indirect immunofluorescence with goat anti-rabbit-Cy5 as a secondary antibody; the actin cytoskeleton was stained with TRITC (tetramethyl rhodamine isothiocyanate)-phalloidin after 5' permeabilization with 0.1% Triton X-100. Images were obtained using a Zeiss Axioconvert 200M inverted microscope equipped with an Ultraview confocal head (Perkin-Elmer) and a krypton argon laser (643-RYP-A01; Melles Griot) with a 100× oil immersion objective lens. Stacks of 0.2 μm distance in z-direction were acquired. Deconvolution was performed in the actin channel with the program Velocity and an appropriate calculated point spread function. An extended focus projection of the three channels is shown.

Construction of strains. Strain M1913, a *ΔsopBEE2 sipA-sptP::aphT* mutant (referred to here as the SipBCD[−] strain), was constructed by P22 transduction (61) of the kanamycin allele of SB245 (*sipABCD sptP::aphT fliGHI::Tn10* [K. Kaniga and J. E. Galan, unpublished data]) into strain Δ4 (15).

Strain M1914, a *ΔsopBEE2 ΔinvG sipA-sptP::aphT* mutant (referred to here as the T1[−] SipBCD[−] strain), was constructed by P22 transduction of strain SB728 (*ΔinvG sipBCDA sptP::aphT* [W. D. Hardt and J. E. Galan, unpublished data]) into strain Δ4. Strains M1910 (*ΔsopBEE2 sipB::aphT*), M1911 (*ΔsopBEE2 sipC::aphT*), and M1912 (*ΔsopBEE2 sipD::aphT*) (see Table 1) were constructed by the P22 transduction of strains SB169, SB220, and SB241 (38), respectively, into the mutant strain M1304 (*ΔsipA ΔsopABEE2 sipA_{M45}*) (60).

S. Typhimurium adhesin mutants were constructed by suicide vector integra-

tion—pGP704 (Amp^r) (47) or pSB377 (Tet^r) (37)—into a gene encoding a structure essential for functional adhesin assembly. Initially, mutants were constructed in the wt strain (SB300) and then P22 transduced (61) into the respective *S. Typhimurium* strain background. PCR primers were designed and used for PCR amplification of the respective gene fragment (data not shown). The PCR fragment was cloned by using HindIII/XbaI into the respective suicide vector. The mutant in *S. Typhimurium* was generated by conjugation of the plasmid from Sm10λpir into the wt strain and selection of suicide vector integrants. Site-specific insertion was verified by PCR (data not shown). Mutations of the *fimA* and *fimH* genes were derived from a large collection of *S. Typhimurium* mutants (56) and P22 transduced into the respective *S. Typhimurium* strain.

RESULTS

Evidence for T1-dependent and -independent binding of *S. Typhimurium* to HeLa cells. We began our study by analyzing T1-dependent and -independent binding of *S. Typhimurium* to HeLa cells. First, we analyzed host cell binding by the Δ4 strain (see Table 1), an isogenic mutant harboring an intact T1 apparatus but lacking the key effectors required for invasion, and by the T1[−] strain (Table 1), lacking a functional type three secretion apparatus. The *S. Typhimurium* wt strain served as a control. Bacteria were grown under T1-inducing conditions, followed by infection of HeLa cells for 12 min, fixation, and staining to differentiate extracellular bacteria bound to the host cell surface (Fig. 1B, blue) and intracellular bacteria (green; compare Materials and Methods). In line with earlier work (42), the Δ4 strain failed to trigger actin rearrangements and to invade but was able to bind to HeLa cells (Fig. 1B). Host cell binding by the T1[−] strain was less pronounced but was still clearly detectable (Fig. 1B, right panel). Our data therefore suggest that *S. Typhimurium* can bind to cells via T1-dependent and T1-independent mechanisms. The Δ4 and T1[−] strains would be suitable tools for studying these mechanisms.

Automated assay for *S. Typhimurium* binding. To study *S. Typhimurium* binding quantitatively, we developed an automated microscopy-based assay (see Materials and Methods). HeLa cells were infected with *S. Typhimurium*, washed, and fixed. After the host cell nuclei (DAPI) and extracellular *S. Typhimurium* (anti-*S. Typhimurium*-lipopolysaccharide [LPS] specific antibodies) were stained, images were acquired by automated microscopy and analyzed by the image analysis routines described in Materials and Methods (Fig. 2A).

To quantify T1-dependent and -independent *S. Typhimurium* binding, HeLa cells were seeded in 96-well dishes and infected for 12 min with the indicated MOIs of the wt, Δ4, or T1[−] strains. Noninfected cells served as a "zero" control ("no bacteria"), which should not yield any HeLa cells with bound bacteria. In most experiments we tested 2-fold dilution series of *S. Typhimurium* starting at an MOI of 1,000. At this MOI, the bacterial concentration in the cellular media is estimated to be 1.67×10^8 per ml; similar bacterial concentrations (10^8 to 10^9 per ml) could be found in the cecum of an *S. Typhimurium*-infected mouse (65).

The *S. Typhimurium* binding assay confirmed the association of the wt and Δ4 strains with cells in a concentration-dependent manner (Fig. 2B). At the highest MOI tested, nearly all cells infected with the wt or Δ4 strain displayed bacterial binding. We did not follow up on the minor additional affinity of the wt strain compared to the Δ4 strain since

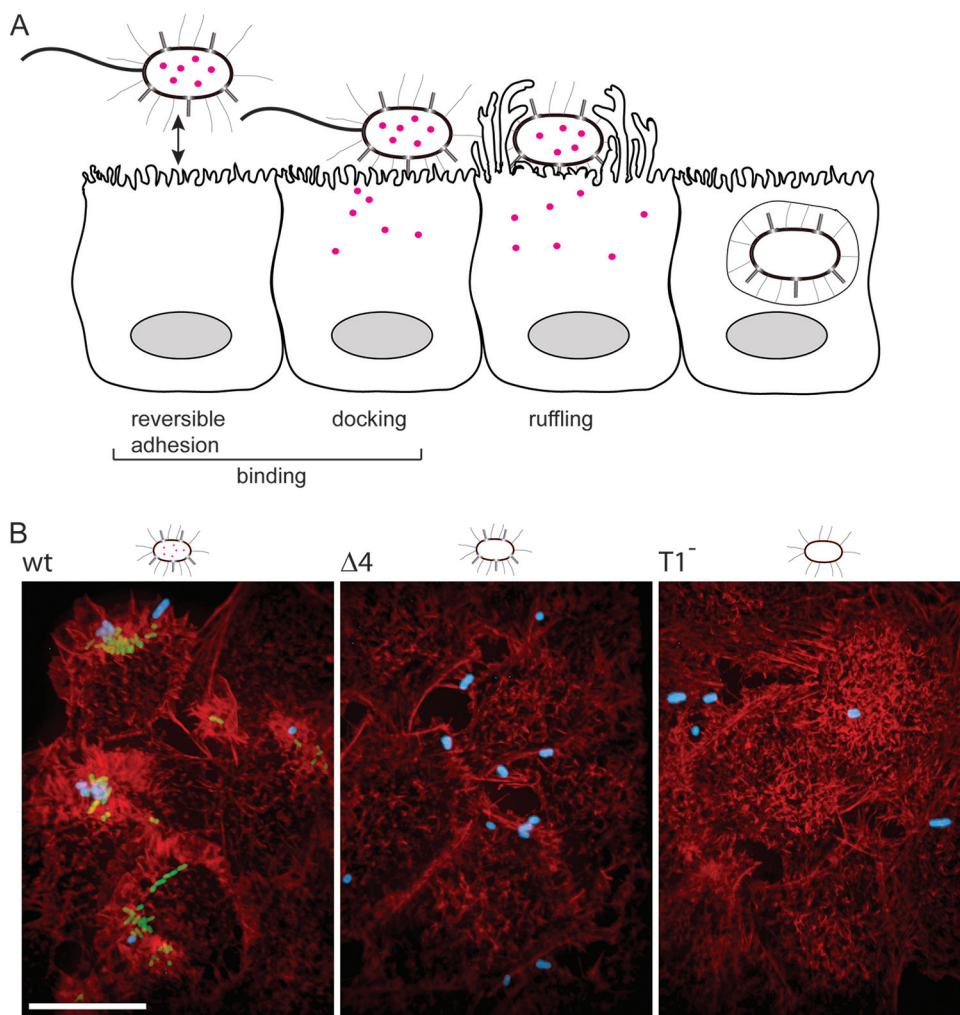


FIG. 1. Binding of *S. Typhimurium* to cells via T1-dependent and -independent mechanisms. (A) Model of *S. Typhimurium* invasion into epithelial cells. In order to invade epithelial cells, *S. Typhimurium* first binds to the surface by an incompletely understood mechanism. It is not known whether reversible adhesion and/or stable association mechanisms might be involved. In the present study, we define “docking” as a stable association (i.e., able to be sustained considerably longer than the invasion process which happens within several minutes). In addition, a “docked” bacterium should be committed to invasion and be able to invade the bound cell without another round of dissociation and association. Bound *S. Typhimurium* then uses a molecular syringe (type three secretion system 1 [T1]) to inject effector proteins (shown in red) into the cytosol of the cell. Some of the effectors activate actin polymerization, which leads to visible ruffles on the surface of the cell. Ruffles are the site of *Salmonella* entry; the bacteria end up in a *Salmonella*-containing vacuole (SCV). (B) Binding of *S. Typhimurium* to cells. HeLa cells were incubated with the indicated *S. Typhimurium* strains at an MOI of 128 for 12 min before fixation and staining. *S. Typhimurium* strains carry plasmid pM965 for constitutive *gfp* expression and are shown in green. Extracellular bacteria are stained in blue due to staining by an anti-*S. Typhimurium* antibody; intracellular bacteria are protected by the membrane from antibody staining. The actin cytoskeleton is shown in red. Scale bar, 50 μ m.

differences in the binding efficiency of these two mutants are most likely attributable to surface changes upon membrane ruffling and/or host cell invasion, which only occurs in the case of the wt strain. In all likelihood the $\Delta 4$ strain represents “T1-dependent binding,” comprising all affinities of *S. Typhimurium* towards the host cell mediated by the T1 apparatus including the translocon but excluding any secondary effects mediated by the translocated effectors. The $\Delta 4$ strain bound more efficiently than the $T1^-$ strain, confirming the important role of T1 for the *S. Typhimurium* cell adhesion. Nevertheless, the $T1^-$ strain also bound to HeLa cells at all MOIs tested. These observations were confirmed with *S. Typhimurium* mutants lacking the translocon proteins SipB, SipC, and SipD of the T1 apparatus (i.e., the SipBCD $^-$ strain, the $T1^-$ SipBCD $^-$

strain, and strains M1910 [Δ sopBEE2 sipB::aphT], M1911 [Δ sopBEE2 sipC::aphT], and M1912 [Δ sopBEE2 sipD::aphT]; Table 1). These proteins are inserted into the host cell membrane and facilitate effector protein translocation. *S. Typhimurium* mutants in any of these proteins bound to HeLa cells with an efficiency equal to that of the $T1^-$ strain (Fig. 2C and data not shown [see reference 42]). These data confirmed the existence of a T1-independent mechanism of HeLa cell binding.

Earlier reports have indicated that the infection efficiencies of some pathogens can vary in their dependence on the host cell density (62). To control this key basic characteristic of our assay, we seeded increasing numbers of HeLa cells and systematically tested the cell density effects on *S. Typhimurium*

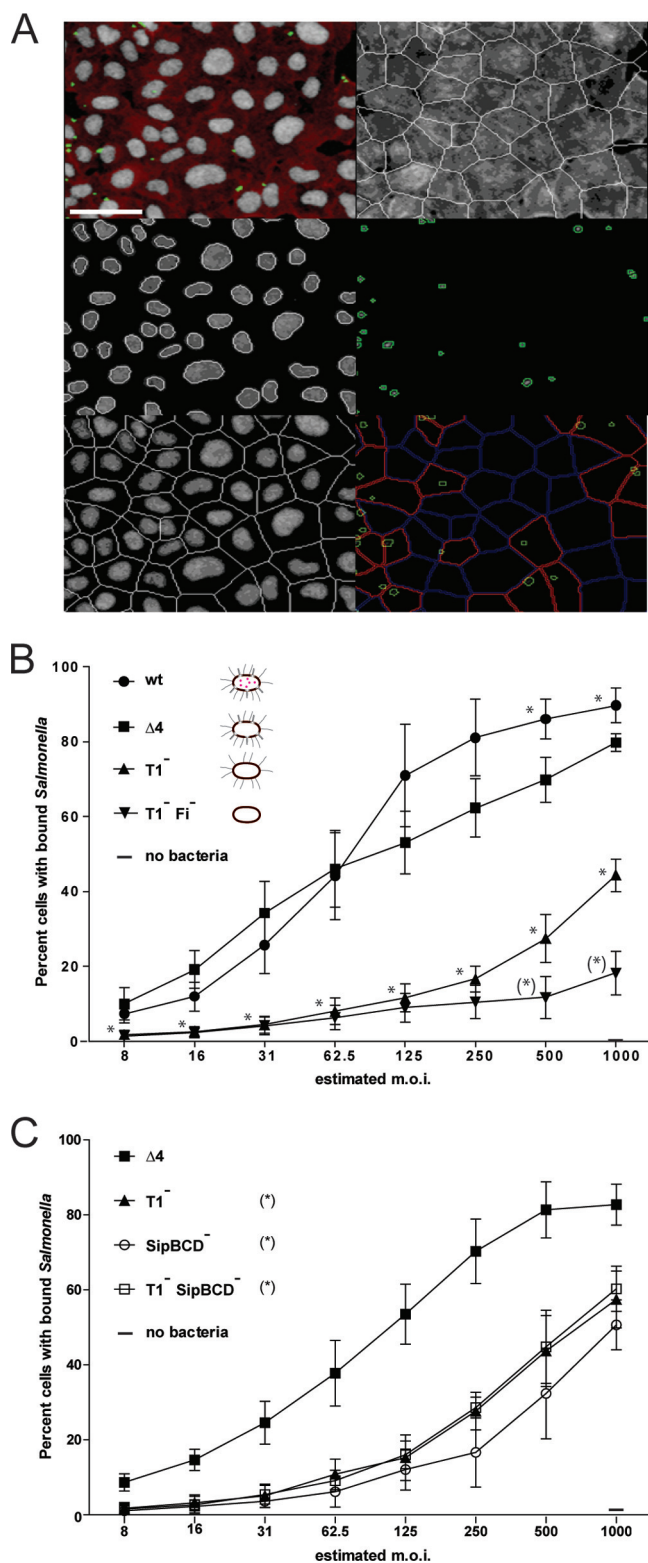


FIG. 2. Automated analysis of *S. Typhimurium* binding. (A) Image analysis strategy to quantify *S. Typhimurium* binding. HeLa cells were incubated with the $\Delta 4$ strain (MOI = 62.5) for 10 min at 37°C. (Upper left panel) Bacteria were stained by an anti-*S. Typhimurium* antibody (green), nuclei were stained with DAPI (gray), and the actin cytoskeleton was stained with TRITC-phalloidin (red). The following panels demonstrate image analysis by the open source program CellProfiler

binding. *S. Typhimurium* binding was expressed as a function of the number of nuclei detected within the four images of each well. As shown in Fig. 3, within the range of 7,000 to 13,000 detected nuclei per well (which are the cell densities used throughout this study), no strong influence of cell number on the measured percentage of binding of the $\Delta 4$ strain (Fig. 3A) and the $T1^-$ strain (Fig. 3B) was detected. Only at very low cell densities we noted a slight increase in *S. Typhimurium* binding, accompanied by a much stronger experimental variability. Since in our experiments the cell numbers detected were usually ca. 10,000 per well and were very similar between wells in the same experiment (data not shown; see Materials and Methods), the experimental variability of cell numbers should have no detectable influence on *S. Typhimurium* binding to HeLa cells, as measured by our assay.

As a further control, we compared *S. Typhimurium* binding to “empty” areas of the well to binding to areas covered by cells (see Materials and Methods). In our calculations, for each well the average number of spots per pixel of the cell area was calculated and defined as “1”. Thereafter, the average number of spots per pixel of the cell free area was calculated and expressed as a fraction of the spot density of the cellular area of the same well. As shown in Fig. 3C, relative *S. Typhimurium* binding to cells was >10-fold stronger than binding to “empty” areas for all strains tested, thus confirming the specificity of our approach.

Fim contributes to $T1^-$ -independent binding. In our experiments, $T1^-$ *S. Typhimurium* strains could still bind to HeLa cells. The mechanism mediating this $T1^-$ -independent binding remained to be established. Known *Salmonella* adhesins include type I fimbriae, Fim (8, 12, 44), plasmid-encoded fimbriae (Pef) (2, 19, 50), long polar fimbriae (Lpf) (1),

and custom algorithms. (Middle left panel) Recognition of nuclei. (Lower left panel) Expansion of the nuclear area to estimate the dimensions of cells. (Upper right panel) Overlay of the cell borders over the actin stain for illustration (the actin stain was not used for the image analysis). (Middle right panel) Detection of spots in the *Salmonella* channel using a threshold that is calculated by comparing infected and noninfected wells. (Lower right panel) Spots and cells are superimposed. Spots are allocated to the cell with the greatest overlap. Cells containing at least one spot are scored as having bound bacteria (red outlines), and cells without *S. Typhimurium* are scored as noninfected (blue outlines). Only a small part of one image is shown. In a typical well, 10,000 cells are identified within the four acquired images. The automated analysis for this well yielded a ratio of infected cells of 49.6%. Scale bar, 100 μ m. (B) *S. Typhimurium* binding to cells via $T1^-$ -dependent and -independent mechanisms. HeLa cells were infected with the different *S. Typhimurium* strains at the indicated MOIs and incubated for 12 min. The medians and standard deviations of five independent experiments are shown. An asterisk (*) indicates a *P* value below 0.05 (Mann-Whitney U test) comparing the tested data point to the $\Delta 4$ strain at the same MOI; an asterisk in parentheses indicates a *P* value below 0.05 comparing the $T1^- Fi^-$ strain to the $T1^-$ strain. Points not marked by an asterisk of the same curve were also tested but did not yield significant *P* values. (C) Efficient *S. Typhimurium* binding requires an intact $T1$ system. HeLa cells were infected as in panel B using either the $\Delta 4$ strain or an isogenic strain carrying a combined knockout of *sipB*, *sipC*, and *sipD* (the $SipBCD^-$ strain). The $T1^-$ strain and a mutant lacking the three translocases as well as $T1$ (the $T1^- SipBCD^-$ strain) are shown for comparison. An asterisk next to the curve indicates a *P* value below 0.05 (Wilcoxon signed-rank test) comparing the tested curve (median values) to the $\Delta 4$ strain.

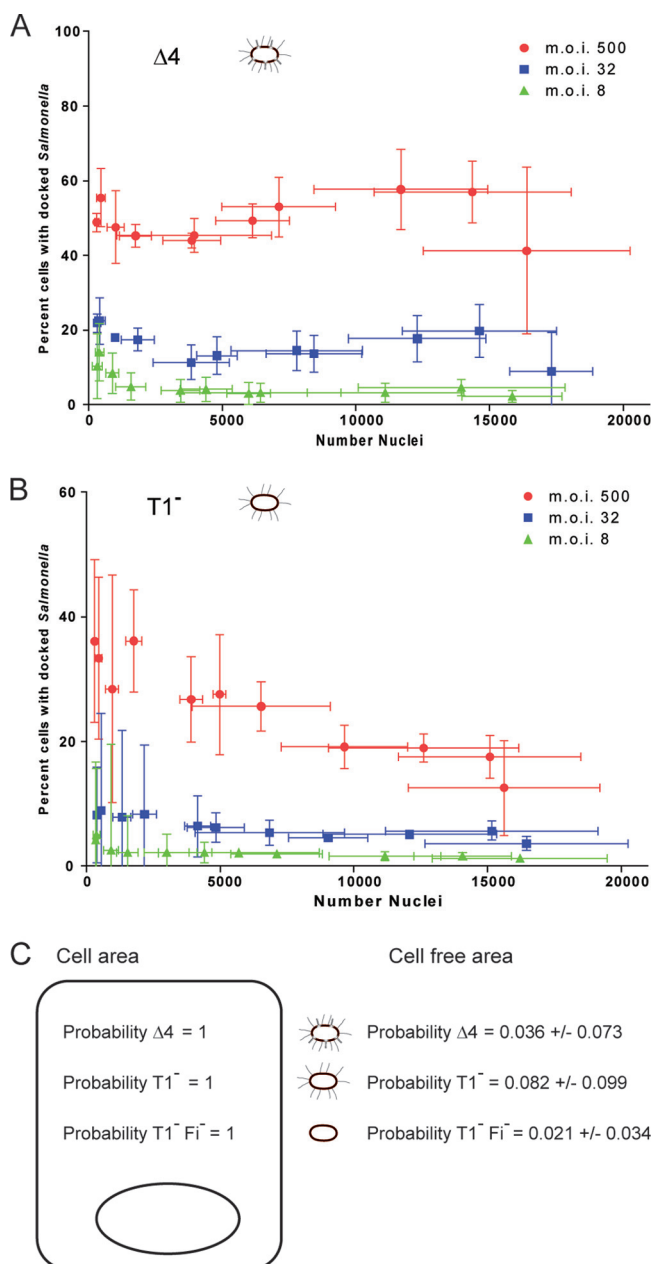


FIG. 3. Fundamental characteristics of the automated *S. Typhimurium* binding assay. (A and B) Increasing numbers of HeLa cells were seeded and infected with the indicated *S. Typhimurium* strain for 12 min. *S. Typhimurium* binding was determined as described in the text and plotted as a function of the number of nuclei detected within the respective well. The data represent the medians of three independent experiments and the standard deviations. (C) Comparison of *S. Typhimurium* binding to HeLa cells and “empty” areas of the same well. The spot density of an empty area of a well was calculated and expressed as a fraction of the spot density of the cell area of the same well. The data represent the medians and standard deviations of 87 to 90 wells at various MOIs from three independent experiments.

thin aggregative fimbriae (Agf) (23, 55, 67), and the adhesin ShdA (39). To test for a role in *S. Typhimurium* binding, we generated knockouts of these adhesins in the background of the $T1^-$ strain. HeLa cells were seeded in 96-well dishes and

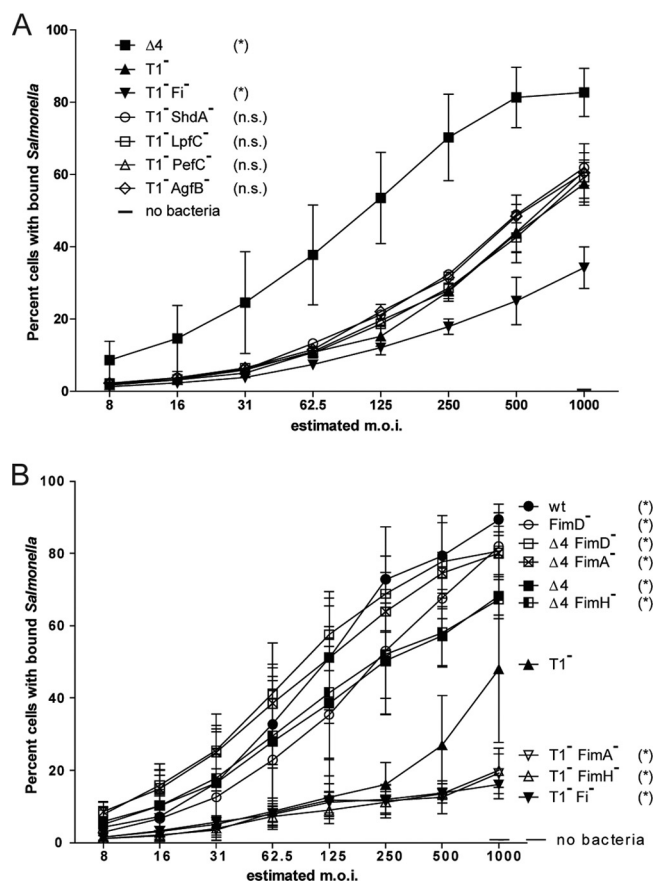


FIG. 4. Role of type I fimbriae for *S. Typhimurium* binding to HeLa cells. HeLa cells were infected with the indicated *S. Typhimurium* strains for 12 min, and binding was analyzed. (A) Deletion mutants of type I fimbriae, *shdA*, long polar fimbriae, plasmid-encoded fimbriae, and thin aggregative fimbriae were analyzed in the background of the $T1^-$ mutant. (B) Deletion mutants of the *fim* operon were generated in the background of the $T1^-$ strain (*fimA*, *fimD*, and *fimH*), the $\Delta 4$ strain (*fimD*, *fimA*, and *fimH*), and the wild-type strain (*fimD*), respectively, and tested for HeLa cell binding. An asterisk next to the curve indicates a *P* value below 0.05 (Wilcoxon signed-rank test) comparing the indicated curve (median values) to the $T1^-$ strain.

infected for 12 min at the indicated MOI, and we analyzed the bacterial binding as described above. The isogenic mutant lacking type I fimbriae (the $T1^- Fi^-$ strain), but none of the other adhesin mutants (M1930 [$\Delta invG$ *pefC*::pSB377], M1929 [$\Delta invG$ *lpfC*::pSB377], M1931 [$\Delta invG$ *agfB*::pSB377], and M1928 [$\Delta invG$ *shdA*::pGP704]) had a reduced binding phenotype compared to the parent $T1^-$ strain (Fig. 4A). This indicated that type I fimbriae contribute to $T1$ -independent HeLa cell binding.

Type 1 fimbriae are produced on the *S. Typhimurium* surface by a chaperone-usher system (35) involving the membrane protein FimD. FimA is the major structural subunit of the fimbrial shaft, and FimH constitutes the terminal fimbrial subunit responsible for substrate binding (26). To confirm the phenotype of the *fimD* deletion, we also tested the deletions of *fimA* and *fimH* in the background of the $T1^-$ strain. As shown in Fig. 4B, deletions of *fimD*, *fimA*, and *fimH* yielded a very similar binding phenotype. Further-

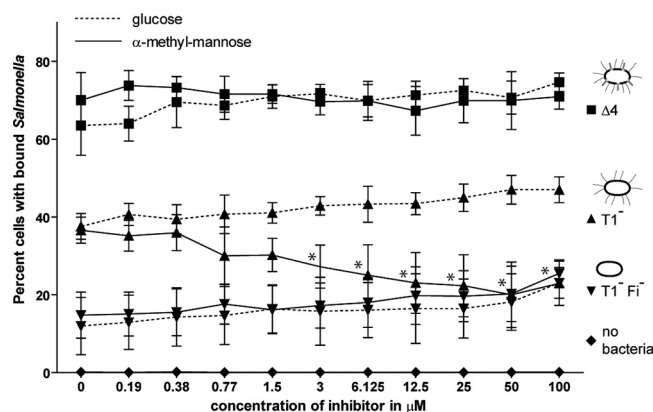


FIG. 5. Inhibition of Fim-mediated binding by α -methyl-mannose. HeLa cells and bacteria were preincubated with the inhibitor α -methyl-mannose or glucose before infection of cells at an MOI of 1,000 for 10 min. The medians and standard deviations of six data points from four independent experiments are shown. An asterisk indicates binding in the presence of α -methyl-mannose that is significantly different compared to binding in the presence of glucose at the same concentration (Mann-Whitney U test). Pairs of data points not marked by an asterisk are not significantly different.

more, deletion of *fimD* in a wt strain background, as well as the deletion of *fimD*, *fimA*, or *fimH* in the $\Delta 4$ strain background had equivalent effects on *S. Typhimurium* binding to HeLa cells. Taken together, these additional *S. Typhimurium* mutants confirmed an important role of type 1 fimbriae for T1-independent binding of *S. Typhimurium* to HeLa cells and suggested only a minor role (if any) of type 1 fimbriae for T1-dependent binding.

It should be noted that even in the absence of both type I fimbriae and T1, some residual *S. Typhimurium* binding remained detectable. This may indicate the presence of at least one additional, unidentified T1-independent binding mechanism. Binding of $T1^- Fi^-$ to cells was also independent of cell numbers, in particular in the range of 7,000 to 13,000 nuclei per well (data not shown). In addition, the spot density within the cellular area was much larger than the density at empty areas of a well (Fig. 3C), suggesting that this residual binding capacity was specific for host cells.

Inhibition of Fim-mediated binding by α -methyl-mannose. Type I fimbriae comprise long extensions from the surface of the bacterium, with FimH at their tips. FimH consists of a pilin domain, anchoring the protein in the fimbrial shaft, and a mannose-binding domain, mediating contact to the targets. α -Methyl-mannose can competitively inhibit Fim-mediated binding (12, 25, 68). Therefore, we used α -methyl-mannose inhibition as an independent assay for verifying the role of type I fimbriae in T1-independent HeLa cell binding.

HeLa cells were seeded in 96-well dishes. Bacteria and cells were preincubated for 10 min with the indicated concentrations of α -methyl-mannose. Glucose, which does not bind to FimH, served as a control for the specificity of the inhibition. The cells were infected for 10 min with the $T1^-$ strain (experimental groups; inhibition expected) or the $T1^- Fi^-$ strain (negative control; no inhibition expected) at MOIs of 1,000 bacteria per cell. Uninfected cells and cells infected with the $\Delta 4$ strain served as additional controls.

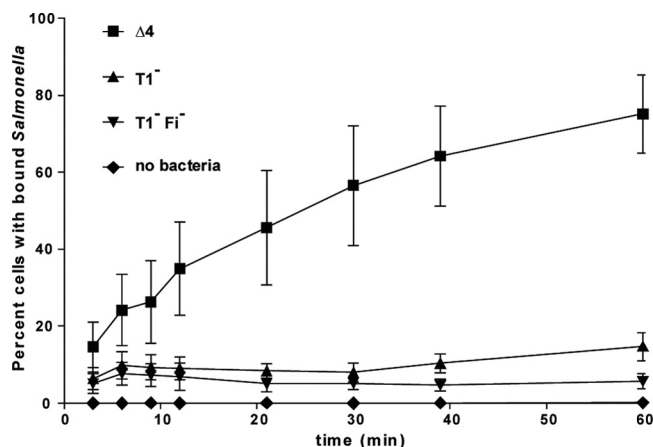


FIG. 6. Time course of the *S. Typhimurium* binding to host cells. HeLa cells were infected with the indicated *S. Typhimurium* strains at an MOI of 62.5 for the indicated times followed by analysis of *S. Typhimurium* binding.

α -Methyl-mannose inhibited the $T1^-$ strain binding in a concentration-dependent manner, whereas glucose at the same concentrations did not show any effect (Fig. 5). At maximum inhibitor concentration, the binding of the $T1^-$ strain was reduced to the level of the $T1^- Fi^-$ strain, indicating that the inhibitor can completely block any type I fimbria-dependent HeLa cell binding. α -Methyl-mannose acted specifically since binding of the $T1^- Fi^-$ strain was not affected. This confirmed that type I fimbriae contribute to T1-independent HeLa cell binding.

Different time courses of T1-mediated and T1-independent binding. We noticed that the T1-mediated and the Fim-mediated host cell binding had different time dependencies (data not shown). We reasoned that this might reveal important new information about the binding mechanism and analyzed the binding of *S. Typhimurium* to HeLa cells as a function of time in a systematic way. HeLa cells were seeded in 96-well dishes and infected with the $\Delta 4$ strain (T1-dependent binding), the $T1^-$ strain (T1-independent binding) or the $T1^- Fi^-$ strain (residual binding) at an MOI of 62.5 bacteria per cell. Noninfected cells served as an additional control. As shown in Fig. 6, host cell binding by the $\Delta 4$ strain increased during the course of the assay. In contrast, the $T1^-$ and $T1^- Fi^-$ strain binding did not increase. Equivalent results were obtained at an MOI of 125 (data not shown). This provided another indication that key parameters of T1-mediated and T1-independent HeLa cell binding may differ.

Both mechanisms of binding can lead to invasion, if ruffling is triggered *in trans*. We next wanted to test whether *S. Typhimurium* binding via T1 and/or type I fimbriae represent an intermediate step of the *S. Typhimurium* invasion process. Neither the $\Delta 4$ strain nor the $T1^-$ strain can invade by itself. Thus, membrane ruffling must be triggered *in trans*. For this purpose, we used a "helper" assay (Fig. 7, left panel). This type of assay has been described before (5, 28). For detection of invaded bacteria, the $\Delta 4$ strain and the $T1^-$ strain were transformed with pM975, a reporter plasmid expressing *gfp* upon entry into the host cell (27). As a helper

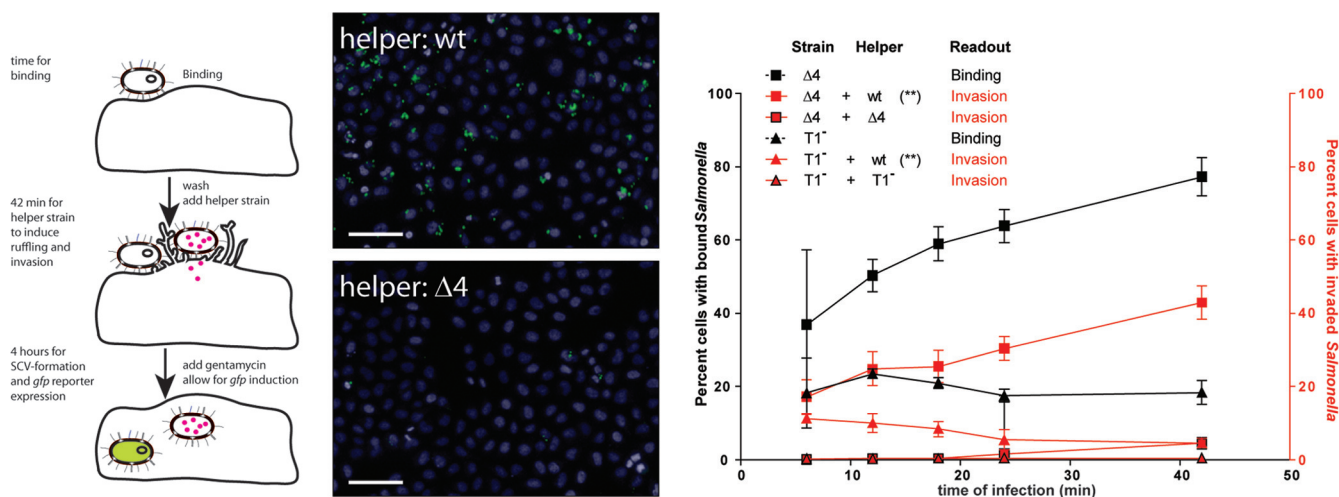


FIG. 7. Bound *S. Typhimurium* can proceed with cellular invasion if ruffling is triggered *in trans*. (Left panel) Scheme of the helper assay. *S. Typhimurium* carrying plasmid pM975 for intracellular GFP production was allowed to bind, followed by washing and addition of the helper strain. The helper strain induces ruffling and internalization of both the previously bound *S. Typhimurium* and the helper bacteria. *S. Typhimurium* was allowed to express *gfp* during another 4 h in medium containing gentamicin for killing extracellular *S. Typhimurium*. Binding of the $\Delta 4$ strain and the $T1^-$ strain (black symbols) was evaluated by anti-LPS staining and automated microscopy. (Middle panel) Microscopy images showing induction of *gfp* expression by the $\Delta 4$ strain (pM975) with the wild-type strain as the helper and no *gfp* expression with the $\Delta 4$ strain as a helper strain. Scale bar, 100 μ m. (Right panel) Quantification of binding at an MOI of 125 for various time points (black dashed lines) and invasion (red lines) of the indicated combinations of noninvasive *S. Typhimurium* and helpers. The helper was tested at various concentrations, and the maximum invasion for each concentration was plotted. For curves marked by two asterisks, a *P* value of less than 0.001 was obtained when invasion in the presence of the wt strain as a helper was compared to the $\Delta 4$ strain or the $T1^-$ strain. For these analyses, all values of the three biological replicates were used in a paired test (Wilcoxon signed-rank test).

strain for effector protein injection and induction of membrane ruffling, an unlabeled *S. Typhimurium* strain (the wt strain; the $\Delta 4$ strain = negative control) strain was used. In our experiments, HeLa cells were first infected with the $\Delta 4$ strain (pM975) or the $T1^-$ strain (pM975), thus allowing for host cell binding. After the indicated time points, the cells were washed and incubated with the helper strain. Thereby, the helper wt strain should facilitate invasion, while the control helper $\Delta 4$ strain should not. During a final incubation step, we allowed for *gfp* expression by all $\Delta 4$ (pM975) or $T1^-$ strain (pM975) bacteria which had successfully entered into the host cell (Fig. 7, middle panel). Finally, the cells were fixed, and bacterial invasion was quantified as described in Materials and Methods. After invasion both the $\Delta 4$ and the $T1^-$ strains remained alive within the host cell and likely ended up in a similar type of *Salmonella*-containing vacuole (SCV) because *gfp* is expressed in both mutants within the hours after invasion (described, below).

When we used the wt strain as a helper, prebound $\Delta 4$ strain (pM975) and $T1^-$ (pM975) strains could enter into the cells. In the absence of a helper (i.e., the negative control) or in cases where we used the noninvasive $\Delta 4$ strain as the helper (i.e., additional negative control), no invasion was detected. The efficiency of the “helped invasion” was dependent on the concentration of both the helper and the reporter strains. Based on our data, we estimated that, under optimal conditions, 50% of the cells with prebound $\Delta 4$ strain (pM975) had invaded bacteria upon addition of the helper strain (Fig. 7, red squares, right panel). More than 50% should not be expected, since the wt strain-triggered ruffles would not exceed 50% of the area of the whole cell, and since the helper wild-type strain would dock

onto cells independently from and at different sites than the $\Delta 4$ strain (pM975). Therefore, we conclude that $T1$ -mediated binding can lead to invasion, if ruffling is triggered *in trans*.

The $T1^-$ strain (pM975) could also proceed to invasion, if the wt strain was provided as the helper strain. Again, we observed that ca. 50% of the measured binding activity could proceed to invasion, if ruffling was triggered *in trans* (Fig. 7, compare black and red triangles). However, in contrast to the $\Delta 4$ strain (pM975), the efficiency of the “helped invasion” did not increase at longer times of binding (Fig. 7, red triangles). We speculate that this might be attributable to the reversible nature of $T1$ -independent HeLa cell binding (this is addressed below). In any case, these data demonstrate that both $T1$ -mediated and $T1$ -independent binding can lead to invasion, if ruffling is triggered *in trans*.

$T1$ mediates irreversible docking, while type I fimbriae mediate reversible adhesion. The data shown above indicated that $T1$ -independent host cell binding might be reversible (“reversible adhesion”), while $T1$ -mediated binding might be irreversible (“docking”; Fig. 1A). To test this hypothesis, we analyzed the rates of dissociation of the $\Delta 4$, $T1^-$, and $T1^-$ Fi^- strains. HeLa cells were seeded in 96-well dishes and infected at the indicated MOIs. At the indicated time points (10-min intervals), unbound bacteria were either washed off and the medium was replaced by fresh medium containing 100 μ M α -methyl-mannose or the cells were fixed. Subsequently, cells and bacteria were stained, and cells harboring bound bacteria were quantified by automated microscopy as described above. The $\Delta 4$ strain remained stably bound to HeLa cells for at least 50 min (Fig. 8). In contrast, the number of cells with bound $T1^-$ and $T1^-$ Fi^- strains decreased in a time-dependent man-

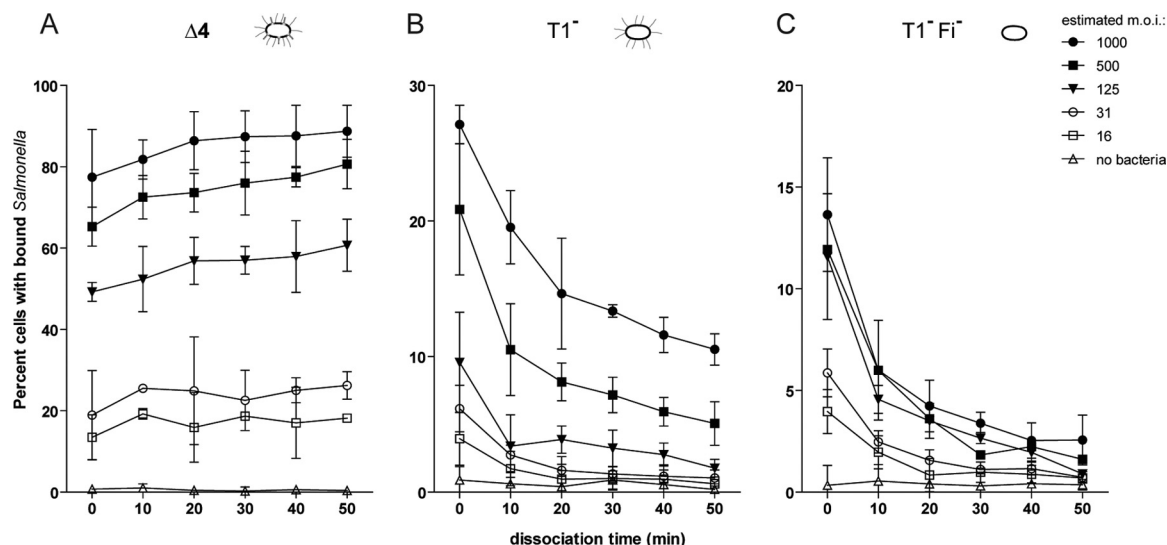


FIG. 8. Dissociation kinetics of T1- and Fim-mediated binding. The indicated *S. Typhimurium* strains were incubated with HeLa cells for 12 min. Plates were subsequently washed and incubated for 10 min at 37°C in medium containing α -methyl-mannose. Several rounds of medium exchange and incubation were performed. The appropriate wells were fixed. Finally, binding was measured as described in the text.

ner. Dissociation did not follow first-order kinetics. However, exponentiation curves could be fitted; the apparent half-life of the T1⁻ strain was 15.2 min and was significantly longer than the apparent half-life of the T1⁻ Fi⁻ strain (10.0 min, $P < 0.0001$). We therefore conclude that T1 mediates irreversible docking of *S. Typhimurium* to cells. In the absence of T1 only a reversible adhesion of *S. Typhimurium* was observed. The slower dissociation rate of the T1⁻ strain (versus the T1⁻ Fi⁻ strain) indicated that type I fimbriae are slowing down the dissociation from the host cell.

DISCUSSION

Our experiments address the binding step of the *S. Typhimurium* invasion process (Fig. 1A). We found that *S. Typhimurium* binds to mammalian cells via T1 (42), type I fimbriae, and at least one additional unknown mechanism. T1-mediated binding ("docking") was irreversible, whereas T1-independent binding was reversible. We could show that type I fimbriae contribute significantly to the latter. If membrane ruffling was triggered *in trans*, both mechanisms of host cell binding could lead to bacterial entry into the host cell.

According to our results, *S. Typhimurium* docked via T1 would be a true intermediate of the invasion process. In the wild-type situation, invasion-mediating effector proteins are translocated by the docked bacterium itself; membrane ruffling and invasion would thus be initiated simultaneously or immediately after docking. However, effector protein injection and the triggering of ruffling can also be provided *in trans*, e.g., by coinfection of a noninvasive strain with the wt helper strain. Our experiments, therefore, allow for a functional separation of docking from the subsequent steps of the invasion process. Invaded bacteria express SPI-2, a hallmark for the intracellular *S. Typhimurium* lifestyle and normal SCV maturation (58), further confirming that host cell docking by the $\Delta 4$ strain can be regarded as a functional intermediate of the invasion pathway.

Importantly, binding via T1 was irreversible, at least for the 50 min of observation, whereas binding in its absence was reversible. This was directly demonstrated by dissociation experiments. In addition, the different time dependencies of T1- and Fim-dependent adhesion can thus be explained: while with T1-dependent adhesion no dissociation occurs, binding can continuously increase over time. In contrast, Fim-dependent adhesion competes with dissociation and a steady state will be reached (Fig. 6).

According to our results, during docking the type three secretion system would therefore have a dual role: it would act as an effector translocating apparatus and as an adhesin. This duplicate function would ensure that *S. Typhimurium*, after injection of effector molecules, could actually benefit from this former activity. Activation of Rho-GTPases and actin polymerization happens with only a slight delay, initiating ruffling and invasion as early as 3 min after docking (data not shown). Therefore, stable association via T1 would ensure that *S. Typhimurium* remains bound to the site of injection for a long enough time.

In previous studies, bacterial binding had been quantified by various methods, including manual counting of cell-associated *S. Typhimurium* (26, 42), studying agglutination of erythrocytes (13), plating assays (3, 11, 40), spectrophotometrically (69), and by flow cytometry (25). In the present study, we introduce a new method to measure *S. Typhimurium* binding onto cells by automated microscopy and automated image analysis. Our assay eliminates manual quantification, which can be limiting in large experiments and could be biased. Our analysis focuses on cellular phenotypes; therefore, bacteria not associated with cells will reliably be excluded. Moreover, since *S. Typhimurium* docking can be quite heterogenous, with large differences between individual cells (49), our focus on cells rather than bacteria prevents individual cells with a high number of bacteria from dominating the picture. Finally, once established, our assay can be performed easily and reliably, even for screening experiments with a large number of data points (not shown). Thus, our assay may be useful for future

analyses of *S. Typhimurium* binding, including systematic screening approaches, and should be easily adaptable for studying host cell binding of other pathogens.

The molecular interactions mediating the stable interaction of cells and T1 are currently unclear. An important clue might be the observation by us and others (42), that mutations of SipB, SipC, and SipD disrupt T1-mediated docking. After host cell contact, SipB and SipC are inserted and integrated into the host cell membrane (9, 32, 57), forming the multimeric translocon, which in turn mediates the translocation of other *S. Typhimurium* effectors. Both SipB and SipC would be able to anchor *S. Typhimurium* at the host cell: SipB can bind to cholesterol (30), which is abundant in the host plasma membrane, whereas SipC is able to bind to and polymerize actin (7, 31). As a stable intermediate of the invasion process is formed, the $\Delta 4$ strain will represent a valuable tool for future studies analyzing the molecular mechanism of docking.

In the absence of T1, the bacterium has other means available to bind to mammalian cells. We could identify type I fimbriae as a second adhesin mediating reversible binding to HeLa cells. The knockout of the fimbrial usher protein FimD, as well as the inhibitor α -methyl-mannose (12), reduced T1-independent *S. Typhimurium* binding to cells. Type I fimbriae are over 1 μ m long and 6 nm wide (41); they consist of the major FimA subunit, as well as additional subunits, with the adhesin FimH at their tip. Fimbriae have been shown to mediate adhesion of *Salmonella* spp. to erythrocytes (68), cells in tissue cultures (3, 4, 11, 25, 26, 40, 68, 69), and the epithelium of the rodent intestine (4, 16). Thereby, *S. Typhimurium* adherence to eukaryotic cells is dependent on the *fimH* gene of the *S. Typhimurium* strain (4). In addition, at least in some instances *fimH* alleles can mediate differential binding to target cells of different species (24). Since fimbria-mediated binding thus depends on bacteria and host properties, it seems possible that T1-independent binding is more pronounced in HeLa cells used by us than Henle-407 cells used in a previous study (42). Recently, GP2 on the surface of M cells has been described as the receptor for *S. Typhimurium* FimH; this receptor was shown to mediate *S. Typhimurium* transport into the Peyer's patch (29). Since M cells can autonomously endocytose bacteria, Fim-mediated reversible binding might be sufficient for ensuring transport into the Peyer's patch, and T1 might be less important in this situation. For other phagocytic cells such as bone marrow-derived immature dendritic cells, it could be shown that *S. Typhimurium* association was dependent on type 1 fimbriae but not on components of T1 (25). Furthermore, *in vivo*, the peristaltic movements of the bowel will generate pronounced shear stress. In an *Escherichia coli* system, shear stress was shown to enhance fimbrial binding via a conformation change of FimH (63). Such a "catch bond" mechanism might therefore enhance the binding and uptake of *S. Typhimurium* by M cells *in vivo*.

Even in the absence of both T1 and Fim, significant residual binding of *S. Typhimurium* was observed. This remaining affinity is clearly higher than the "background." The *S. Typhimurium* genome contains more than a dozen fimbrial operons (*agf*, *bcf*, *fim*, *lpf*, *pef*, *saf*, *stb*, *stc*, *std*, *stf*, *sth*, *sti*, and *stj*) (14, 54). In our experiments, individual knockdowns of four adhesins other than type I fimbriae in the background of the T1⁻ strain did not affect binding. This is in line with previous data showing that many *S. Typhimurium* adhesins are only induced *in vivo* (36).

A function of SiiE, another invasion enhancing adhesin en-

coded on *Salmonella* pathogenicity island 4, can probably be ruled out as well. This adhesin enhances invasion of the apical surface of polarized cells but does not affect *S. Typhimurium* binding to HeLa cells (20). Nevertheless, we cannot rigorously exclude the role of any adhesin not included in the present study. Alternatively, biophysical forces, including electrostatic interactions (33), might contribute to host cell binding. The mechanisms mediating host cell binding under various conditions and their interplay with biophysical forces remain a fascinating field of infection biology. The analysis of T1⁻ Fim⁻ binding to HeLa cells by the automated microscopy assay described here might provide a valuable experimental system for this type of research and for identifying additional mechanisms contributing to HeLa cell binding.

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